WEST Search History

#14
9/23/02

DATE: Monday, September 23, 2002

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT,PGPB,JPAB,EPAB; PLUR=YES; OP=OR			
L4	L3 and ns5b	3	L4
L3	L2 AND crystal	2525	L3
L2	L1 and @pd<20000630	20306	L2
L1	polymerase	35725	L1

END OF SEARCH HISTORY

Set	Items	Description
S1	190207	POLYMERASE
S2	11815	S1 AND INHIBITORS
S3	15	S2 AND NS5B
S4	2	S3 AND DESIGN
S5	4	S3 NOT PY=>1999
S6	6	S3 NOT PY=>2000

#14

9/23/02

6/9/1
DIALOG(R)File 155:MEDLINE(R)

10[illegible] 99137[illegible] PMID: 10094969

Expression of hepatitis C virus NS5B protein: characterization of its RNA polymerase activity and RNA binding.

Ishii K; Tanaka Y; Yap C C; Aizaki H; Matsuura Y; Miyamura T

Department of Virology II, National Institute of Infectious Diseases (Former National Institute of Health), Tokyo, Japan.

Hepatology (Baltimore, Md.) (UNITED STATES) Apr 1999, 29 (4) p[illegible]-[illegible], ISSN 0270-9139 Journal Code: 8302946

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The nonstructural protein 5B (**NS5B**) of hepatitis C virus (HCV) is considered to possess RNA-dependent RNA **polymerase** (RdRp) activity and to play an essential role for the viral replication. In this study, we expressed the **NS5B** protein of 65 kd by a recombinant baculovirus. With the highly purified **NS5B** protein, we established an in vitro system for RdRp activity by using poly(A) as a template and a 15-mer oligo(U) (oligo(U)15) as a primer. Optimal conditions of temperature and pH for primer-dependent **polymerase** activity of the **NS5B** were 32 degrees C and pH 8.0. The addition of 10 mmol of Mg2+ increased the activity. The importance of three motifs conserved in RdRp among other positive-strand RNA viruses was confirmed by introduction of an Ala residue to every amino acid of the motifs by site-directed mutagenesis. All mutants lost RdRp activity, but retained the RNA binding activity, except one mutant at Thr287/Asn291. Deletion mutant analysis indicated that the N-terminal region of **NS5B** protein was critical for the RNA binding. Inhibition of RdRp activity by (-)beta-L-2', 3'-dideoxy-3'-thiacytidine 5'-triphosphate (3TC; lamivudine triphosphate) and phosphonoacetic acid (PAA) was observed after screening of nucleoside analogs and known **polymerase inhibitors** . These data provide us not only important clues for understanding the mechanism of HCV replication, but also a new target of antiviral therapy.

Tags: Animal; Support, Non-U.S. Gov't

Descriptors: RNA-Directed RNA **Polymerase** --metabolism--ME; *RNA --metabolism--ME; *RNA Replicase--metabolism--ME; *Viral Nonstructural Proteins--metabolism--ME; Baculoviridae--genetics--GE; Cell Line; Mutagenesis, Site-Directed; Point Mutation; Protein Binding--genetics--GE; RNA Replicase--genetics--GE; RNA Replicase--isolation and purification--IP ; Spodoptera; Transfection; Viral Nonstructural Proteins--genetics--GE; Viral Nonstructural Proteins--isolation and purification--IP

CAS Registry No.: 0 (NS-5 protein, hepatitis C virus); 0 (Viral Nonstructural Proteins.; 63231-63-0 (RNA)

Enzyme No.: EC 2.7.7.48 (RNA Replicase); EC 2.7.7.6 (DNA-Directed RNA **Polymerase**)

Record Date Created: 19990520

6/9/2
DIALOG(R)File 155:MEDLINE(R)

10[illegible]18 99099080 PMID: 9832374

Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in Escherichia coli.

Ferrari E; Wright-Minogue J; Fang J W; Baroudy B M; Lau J Y; Hong Z

Antiviral Therapy, Schering-Plough Research Institute, Kenilworth, New Jersey 07033-0539, USA.

Journal of virology (UNITED STATES) Feb 1999, 73 (2) p1649-54, ISSN 0022-538X Journal Code: 0113724

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

Production of soluble full-length nonstructural protein 5B (**NS5B**) of

hepatitis C virus (HCV) has been shown to be problematic and requires the addition of salts, glycerol, and detergents. In an effort to improve the solubility of **NS5B**, the hydrophobic C terminus containing 21 amino acids was removed, yielding a truncated **NS5B** (NS5BDeltaCT) which is highly soluble and monodispersed in the absence of detergents. Fine deletional analysis of this region revealed that a four-leucine motif (LLLL) in the hydrophobic domain is responsible for the solubility profile of the full-length **NS5B**. Enzymatic characterization revealed that the RNA-dependent RNA **polymerase** (RdRp) activity of this truncated **NS5B** was comparable to those reported previously by others. For optimal enzyme activity, divalent manganese ions (Mn^{2+}) are preferred rather than magnesium ions (Mg^{2+}), whereas chelating ions inhibit the RdRp activity. Ribotoxin, a known poliovirus 3D RdRp inhibitor, inhibited HCV **NS5B** RdRp in a dose-dependent manner. Kinetic analysis revealed that HCV **NS5B** has a rather low processivity compared to those of other known polymerases.

Tags: Human; Support, Non-U.S. Gov't

Descriptors: *Hepacivirus--enzymology--EN; *RNA Replicase--metabolism--ME; *Viral Nonstructural Proteins--metabolism--ME; Amino Acid Sequence; Cations, Divalent; Enzyme **Inhibitors** --pharmacology--ED; Escherichia coli; Kinetics; Molecular Sequence Data; RNA Replicase--antagonists and **inhibitors** --AI; RNA Replicase--genetics--GE; RNA Replicase--isolation and purification--IP; Recombinant Fusion Proteins--genetics--GE; Recombinant Fusion Proteins--isolation and purification--IP; Recombinant Fusion Proteins--metabolism--ME; Sequence Deletion; Solubility; Viral Nonstructural Proteins--antagonists and **inhibitors** --AI; Viral Nonstructural Proteins--genetics--GE; Viral Nonstructural Proteins--isolation and purification--IP

CAS Registry No.: 0 (Cations, Divalent); 0 (Enzyme Inhibitors); 0 (Metals); 0 (NS-5 protein, hepatitis C virus); 0 (Recombinant Fusion Proteins); 0 (Viral Nonstructural Proteins); 67-99-2 (Ribotoxin)

Enzyme No.: EC 2.7.7.48 (RNA Replicase)

Record Date Created: 19990218

6/9/3

DIALOG(R)File 155:MEDLINE(R)

10979062 98414643 PMID: 9740782

Biochemical and kinetic analyses of NS5B **RNA-dependent RNA** polymerase **of the hepatitis C virus.**

Lohmann V; Roos A; Korner F; Koch J O; Bartenschlager R

Institute for Virology, Johannes-Gutenberg University Mainz, Obere Zahlbacher Strasse 67, Mainz, 55131, Germany.

Virology (UNITED STATES) Sep 15 1998, 249 (1) p108-18, ISSN 0042-6822 Journal Code: 0110674

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The biochemical properties of the RNA-dependent RNA **polymerase** (RdRp) of the hepatitis C virus were analyzed. A hexahistidine affinity-tagged **NS5B** fusion protein was expressed with recombinant baculoviruses in insect cells and purified to near homogeneity. Enzymatic activity of the purified protein was inhibited by KCl or high concentrations of NaCl and was absolutely dependent on Mg^{2+}, which could be replaced by Mn^{2+}. **NS5B** was found to be processive and able to copy long heteropolymeric templates with an elongation rate of 150-200 nucleotides/min at 22 degrees C. Kinetic constants were determined for all four nucleoside triphosphates and different templates. In case of a heteropolymeric RNA template corresponding to the last 319 nucleotides of the hepatitis C virus genome, Km values for UTP, GTP, ATP, and CTP were approximately 1.0, approximately .5, approximately 10, and approximately 0.3 microM, respectively. The profile of several **inhibitors** of RdRp activity and substrate analogs indicated that the enzyme has a strong preference for ribonucleoside 5'-triphosphates and that it closely resembles 3Dpol of picornaviruses. Copyright 1998 Academic Press.

Tags: Human; Support, Non-U.S. Gov't

Descriptors: *Hepacivirus--enzymology--EN; *Viral Nonstructural Proteins --metabolism--ME; Kinetics; RNA Replicase--metabolism--ME; Substrate Specificity
CAS Registry No.: 0 (NS-5 protein, hepatitis C virus); 0 (Viral Nonstructural Proteins)
Enzyme No.: EC 2.7.7.48 (RNA Replicase)
Record Date Created: 19981011

6/9/4
DIALOG(R)File 155:MEDLINE(R)

09965294 98340240 PMID: 9775642
Candidate targets for hepatitis C virus-specific antiviral therapy.
Bartenschlager R
Institute for Virology, University of Mainz, Germany. bartnsch@mailmza.zdv.uni-mainz.de
Intervirology (SWITZERLAND) 1997, 40 (5-6) p378-93, ISSN 0300-5526 Journal Code: 0364265
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS
The hepatitis C virus (HCV) was identified as the major causative agent of posttransfusion and community-acquired non-A, non-B hepatitis throughout the world. It is an enveloped virus with a plus-strand RNA genome encoding a polyprotein of about 3,010 amino acids. This polyprotein is cleaved co- and posttranslationally into mature viral proteins by host cell signal peptidases and 2 viral enzymes designated the NS2-3 proteinase and the NS3/4A proteinase complex. It is assumed that virus replication takes place in a membrane-associated complex containing at least 2 viral enzymatic activities: the NS3 nucleoside triphosphatase (NTPase)/helicase and the **NS5B** RNA-dependent RNA **polymerase** (RdRp). Based on their important role for the viral life cycle and the wealth of information available for related cellular and viral proteins, the NS3/4A serine-type proteinase complex, the NS3 NTPase/helicase and the **NS5B** RdRp are the most attractive targets for development of HCV-specific antiviral therapies. This review will summarize our current knowledge about structure and function of these proteins and describe approaches pursued to identify effective antiviral compounds. (122 Refs.)
Tags: Human; Support, Non-U.S. Gov't
Descriptors: *Hepacivirus--physiology--PH; *Virus Replication --drug effects--DE; Cysteine Endopeptidases--metabolism--ME; DNA Helicases --antagonists and **inhibitors** --AI; Endopeptidases--metabolism--ME; Hepacivirus--metabolism--ME; Models, Molecular; RNA Replicase--antagonists and **inhibitors** --AI; Viral Nonstructural Proteins--metabolism--ME
CAS Registry No.: 0 (NS-5 protein, hepatitis C virus); 0 (NS3 protein, hepatitis C virus); 0 (NS4 protein, hepatitis C virus); 0 (Viral Nonstructural Proteins)
Enzyme No.: EC 2.7.7.48 (RNA Replicase); EC 3.4.- (Endopeptidases); EC 3.4.22.- (Cysteine Endopeptidases); EC 3.4.22.- (NS2-3 protease); EC 5.99.- (DNA Helicases)
Record Date Created: 19981001

6/9/5
DIALOG(R)File 155:MEDLINE(R)

09870558 98281516 PMID: 9620206
Expression of recombinant hepatitis C virus non-structural protein 5B in Escherichia coli.
Al R H; Xie Y; Wang Y; Hagedorn C H
Department of Medicine, Genetics Program of the Winship Cancer Center, Emory University School of Medicine, Atlanta, GA 30322, USA.
Virus research (NETHERLANDS) Feb 1998, 53 (2) p141-9, ISSN 0168-1702 Journal Code: 8410979
Contract/Grant No.: AI41424; AI; NIAID

Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Subfile: INDEX MEDICUS

The hepatitis C virus (HCV) represents a major public health problem that can produce liver failure and hepatocellular carcinoma in chronically infected patients. Our goal was to express the HCV non-structural protein 5B (**NS5B**) protein of HCV genotype 1a in Escherichia coli and initiate studies of its role in HCV genomic replication. In this report we demonstrate that a recombinant **NS5B** protein with an amino terminal sequence of ASMSYSWTG has RNA-dependent RNA **polymerase** (RDRP) activity. This recombinant enzyme was active in poly(U) **polymerase** assays and produced template-sized RNA products when globin mRNA was used as a template. The **polymerase** activity of recombinant **NS5B** was primer-dependent and was active for at least 6 min of incubation at 30 degrees C. Deletion of the carboxyl terminal region of HCV **NS5B** resulted in a loss of RDRP activity indicating that the enzymatic activity observed was due to the full-length recombinant enzyme. Recombinant **NS5B** (RDRP) should assist in understanding the mechanism of HCV replication and the identification of specific enzyme **inhibitors** .

Tags: Human; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

Descriptors: *Escherichia coli--metabolism--ME; *Hepacivirus--enzymology --EN; *RNA Replicase--biosynthesis--BI; *Viral Nonstructural Proteins --biosynthesis--BI; Gene Expression; Globins--genetics--GE; Hepacivirus --genetics--GE; Mutagenesis; Poly U--metabolism--ME; RNA Replicase --genetics--GE; RNA, Messenger--metabolism--ME; Recombinant Fusion Proteins --biosynthesis--BI; Recombinant Fusion Proteins--genetics--GE; Templates; Viral Nonstructural Proteins--genetics--GE

CAS Registry No.: 0 (NS-5 protein, hepatitis C virus); 0 (RNA, Messenger); 0 (Recombinant Fusion Proteins); 0 (Viral Nonstructural Proteins); 27416-86-0 (Poly U); 9004-22-2 (Globins)

Enzyme No.: EC 2.7.7.48 (RNA Replicase)

Record Date Created: 19960610

6/9/6

DIALOG(R)File 155:MEDLINE(R)

08297686 95056043 PMID: 7966606

Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics.

Lin C; Pragai B M; Grakoui A; Xu J; Rice C M

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093.

Journal of virology (UNITED STATES) Dec 1994, 68 (12) p8147-57, ISSN 0022-538X Journal Code: 0113724

Contract/Grant No.: CA57973; CA; NCI

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

The hepatitis C virus H strain (HCV-H) polyprotein is cleaved to produce at least 10 distinct products, in the order of NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A- **NS5B** -COOH. An HCV-encoded serine proteinase activity in NS3 is required for cleavage at four sites in the nonstructural region (3/4A, 4A/4B, 4B/5A, and 5A/5B). In this report, the HCV-H serine proteinase domain (the N-terminal 181 residues of NS3) was tested for its ability to mediate trans-processing at these four sites. By using an NS3-5B substrate with an inactivated serine proteinase domain, trans-cleavage was observed at all sites except for the 3/4A site. Deletion of the inactive proteinase domain led to efficient trans-processing at the 3/4A site. Smaller NS4A-4B and NS5A-5B substrates were processed efficiently in trans; however, cleavage of an NS4B-5A substrate occurred only when the serine proteinase domain was coexpressed with NS4A. Only the N-terminal 35 amino acids of NS4A were required for this activity. Thus, while NS4A appears to be absolutely required for trans-cleavage at the

4E/5A site, it is not an essential cofactor for serine proteinase activity. To begin to examine the conservation (or divergence) of serine proteinase-substrate interactions during HCV evolution, we demonstrated that similar trans-processing occurred when the proteinase domains and substrates were derived from two different HCV subtypes. These results are encouraging for the development of broadly effective HCV serine proteinase **inhibitors** as antiviral agents. Finally, the kinetics of processing in the nonstructural region was examined by pulse-chase analysis. NS3-containing precursors were absent, indicating that the 2/3 and 3/4A cleavages occur rapidly. In contrast, processing of the NS4A-5B region appeared to involve multiple pathways, and significant quantities of various polyprotein intermediates were observed. **NS5B**, the putative RNA **polymerase**, was found to be significantly less stable than the other mature cleavage products. This instability appeared to be an inherent property of **NS5B** and did not depend on expression of other viral polypeptides, including the HCV-encoded proteinases.

Tags: Animal; Comparative Study; Human; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

Descriptors: *Hepacivirus--enzymology--EN; *Protein Processing, Post-Translational; *Viral Nonstructural Proteins--metabolism--ME; Amino Acid Sequence; Base Sequence; Binding Sites; Cell Line; Cercopithecus aethiops; DNA Primers; Hamsters; Kidney; Kinetics; Molecular Sequence Data; **Polymerase** Chain Reaction; Restriction Mapping; Sequence Homology, Amino Acid; Tumor Cells, Cultured; Viral Nonstructural Proteins--biosynthesis--BI

CAS Registry No.: 0 (DNA Primers); 0 (NS3 protein, hepatitis C virus); 0 (Viral Nonstructural Proteins)

Record Date Created: 19941212

Candidate targets for hepatitis C virus-specific antiviral therapy

Intervirology; Basel; Sep-Dec 1997; Ralf Bartenschlager;

Volume: 40
Issue: 5/6
Start Page: 378
ISSN: 03005526
Full Text:

[Headnote]
Key Words
Hepatitis C virus
HCV chemotherapy
HCV proteinase inhibitors
HCV-specific antivirals
NS3/4A proteinase complex of HCV
HCV NS3 helicase
HCV NS5B polymerase
HCV replication

[Headnote]
Summary

[Headnote]
The hepatitis C virus (HCV) was identified as the major causative agent of posttransfusion and community-acquired non-A, non-B hepatitis throughout the world. It is an enveloped virus with a plus-strand RNA genome encoding a polyprotein of about 3,010 amino acids. This polyprotein is cleaved co- and posttranslationally into mature viral proteins by host cell signal peptidases and 2 viral enzymes designated the NS2-3 proteinase and the NS3/4A proteinase complex. It is assumed that virus replication takes place in a membrane-associated complex containing at least 2 viral enzymatic activities: the NS3 nucleoside triphosphatase (NTPase)/helicase and the NS5B RNA-dependent RNA polymerase (RdRp). Based on their important role for the viral life cycle and the wealth of information available for related cellular and viral proteins, the NS3/4A serine-type proteinase complex, the NS3 NTPase/helicase and the NS5B RdRp are the most attractive targets for development of HCV-specific antiviral therapies. This review will summarize our current knowledge about structure and function of these proteins and describe approaches pursued to identify effective antiviral compounds.

Introduction

The hepatitis C virus (HCV) is the major cause of transfusion-associated and sporadic non-A, non-B hepatitis cases worldwide [for reviews, see ref 1, 2]. Most if not all infections become chronic and lead to various clinical outcomes including an inapparent carrier state with normal or almost normal liver functions, acute hepatitis and in about 50% of cases a chronic hepatitis. Approximately 20% of chronic cases develop liver cirrhosis which leads to liver failure in about 25% of cirrhotic cases. Furthermore, patients with chronic HCV infections, in particular those which lead to cirrhosis, are at high risk to develop a hepatocellular carcinoma, and HCV is the second most common etiologic agent in the development of this disease [1, 2].

HCV infections are found throughout the world with prevalences among volunteer blood donors ranging between 0.4% (USA, UK) and 2% (Japan, Taiwan) to more than 14% (Egypt) [2]. The virus is transmitted primarily by the parenteral route and many HCV-infected individuals are intravenous drug users or recipients of blood or blood products. Although with the implementation of serological tests to monitor antibodies against HCV the prevalence of transfusion-associated hepatitis has decreased markedly, prevalences still are high because 4050% of infections are not associated with identified risk factors. However, about two thirds of these 'sporadic' infections have a low socio-economic background often associated with some other type of high-risk behaviour like contact with a sexual partner who used intravenous drugs.

Chronic HCV infections so far can only be treated with interferon-alpha. However, in only about 40% of

treated patients do serum alanine aminotransferase (an indicator for liver cell damage) levels decrease to normal. Unfortunately, after cessation of therapy, about 70% of these responders relapse and only 25% of patients show longterm normalization of serum alanine aminotransferase levels [3, 4]. Although this number can possibly be increased in combination therapy with the purine nucleoside analogue ribavirin [5-8], preliminary data indicate that even in this case only about 40% of treated patients show a long-term response [9] demonstrating the need for more effective therapy against chronic HCV infections.

This review will briefly summarize some aspects on the molecular biology of HCV. It will focus on polyprotein processing by the NS3 proteinase and RNA replication and describe approaches how some of the viral enzymes involved in these processes may be used as targets for an effective antiviral therapy.

The Infectious Agent

Studies on the HCV so far have been hampered by the relatively low virus titres in infectious sera, the low expression levels of viral antigens in infected tissues, the lack of an infectious cDNA clone or tissue culture systems to propagate the virus and the lack of a convenient animal model. Therefore, most of our knowledge about virus structure, genome organization and function of viral proteins is derived from a limited number of experimental infections of chimpanzees, which are the only animal model available [10], molecular cloning of HCV cDNAs [11-IS], recombinant expression systems and the analogy to other related viruses. From these analyses, it is known that HCV is an enveloped virus with a plus-stranded RNA genome about 9,600 nucleotides in length. The genome carries a single long open reading frame encoding a polyprotein of about 3,010 amino acids which is cleaved co- and posttranslationally into mature viral proteins (fig. 1). The open reading frame is flanked by 5' and 3' untranslated regions about 340 and 230 nucleotides in length, respectively, important for translation of the polyprotein and RNA replication [16-23].

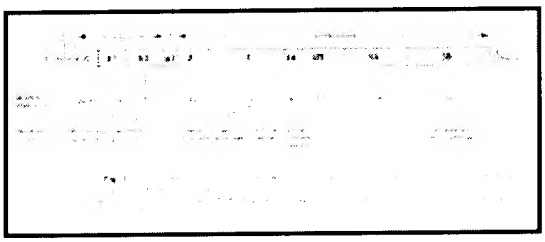

Fig. 1.

Recombinant expression systems have been used to identify HCV polyprotein cleavage products and to delineate their arrangement within the genome. Their order is (from the amino to the carboxy terminus): NH2-C-E1-E2p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (fig. 1) [24-30]. Specific functions can be ascribed to most of these proteins. The C-protein, a basic RNA-binding protein, most likely is the major constituent of the nucleocapsid [31, 32]. E1 and E2 are heavily glycosylated transmembrane proteins embedded into the viral lipid envelope [33-36]. Next to these structural proteins is a small hydrophobic peptide designated p7 the function of which so far is not known. Nonstructural proteins (NS) 2-SB probably are not constituents of the virus particle but are rather involved in polyprotein processing and RNA replication. NS2 and the amino terminal domain of NS3 constitute the NS2-3 proteinase cleaving the NS2/3 junction most likely in an intramolecular reaction [37-39]. The same amino terminal NS3 domain is a serine-type proteinase required for cleavage at all other sites within the NS region [25, 28, 39-43]. In addition to this function, the NS3 protein has a nucleoside triphosphatase (NTPase)/ helicase activity located in the carboxy-terminal two thirds of the molecule [44-48]. NS4A is a cofactor of the NS3 proteinase modulating the activity of this enzyme [49-52]. The functions of NS4B and NS5A so far are not known. NS5B is an RNA-dependent RNA polymerase (RdRp) [53-55].

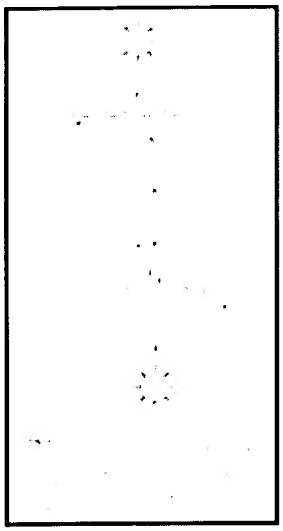

Fig. 2.

A similar genome organization is found with the closely related flaviviruses, the animal pathogenic pestiviruses and the recently cloned GB viruses, in particular the GBV-B isolate [56-58]. All these viruses are enveloped plus-strand RNA viruses with a genome containing a single long open reading frame flanked by 5' and 3' untranslated regions. In addition, a significant coincidence between the hydrophobicity profiles of the viral polyproteins is found as well as short colinear regions of sequence homologies within the polyproteins corresponding in part to proteins with analogous functions [59]. Consequently, HCV is now classified as the separate genus hepatitis C virus together with the genera flaviviruses and pestiviruses in the family Flaviviridae [60]. Although the GB viruses have not been classified so far, they can be regarded as novel members of the same family.

The HCV Replication Cycle

Hepatocytes appear to be a major site of HCV replication. In addition, there is growing evidence that HCV can also replicate within mononuclear cells or establish a latent infection in these cells which can be activated by certain stimuli [61]. Thus, a lymphotropism of HCV could be relevant to the establishment of chronic infections and explain the frequently observed reinfection of transplanted liver grafts [62, 63].

Due to the limitations described above, currently only a speculative model on the replication of HCV can be drawn (fig. 2). After binding to the target cell, which may involve interaction between a host cell receptor and the viral E proteins, HCV penetrates the cell and the viral plus-strand RNA is released from the nucleocapsid into the cytoplasm of the host cell. This RNA is translated and the resulting polyprotein is cleaved co- and posttranslationally by cellular and two viral proteinases into mature viral proteins. Translation occurs at the rough endoplasmic reticulum and viral proteins remain tightly associated with intracellular membranes probably forming a membrane-associated replication complex into which at least the NS3 helicase and the NS5B polymerase are incorporated. Within this complex, the viral RNA plus-strand is copied into several minus-strand RNAs which in turn serve (1) as template for synthesis of progeny plus-strand RNAs, (2) as mRNAs for translation of viral proteins or (3) are incorporated into the nucleocapsid. Circumstantial evidence suggests that nucleocapsids acquired the envelope by budding into the lumen of intracellular vesicles. In this case, HCV particles would be released from the cell via the constitutive secretory pathway.

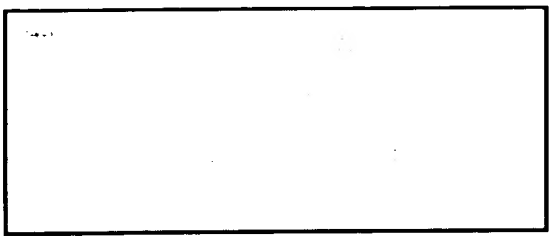

Table 1.

In principle, each step in the viral life cycle represents a potential target for antiviral therapy. However, most of these steps are poorly understood or hypothetical, and the viral and cellular proteins involved are not known. In this respect, the most attractive targets are the NS3 serine-type proteinase and the 2 recently characterized components of the viral replicase: the NS3 NTPase/helicase and the NSSB RdRp. I will focus the rest of this article to function and structure of these enzymes and describe different strategies currently pursued to develop effective inhibitors.

Processing of the Viral Polyprotein Precursor

Cell-free transcription/translation and transient expression of HCV genome fragments in cell culture have been used to characterize processing of the viral polyprotein. As deduced from hydrophobic sequences preceding the cleavage sites and the dependence on microsomal membranes, the C-NS2 region is processed by host signal peptidases cleaving at the C/El, El/E2, E2/p7, p7/NS2 junctions (table 1) [24, 29, 30, 42, 64]. The production of processing intermediates, most notably an E2-p7-NS2 protein, indicates that not all cleavages within the structural region are cotranslational [29, 30, 35, 65]. Furthermore, a second posttranslational cleavage close to the carboxy terminus of the C protein takes place removing the El signal sequence [31, 66].

In contrast to the structural region which is processed exclusively by host cell enzymes, the NS region is cleaved by 2 viral proteinases (table 1). Of these, the most specialized and least understood is the NS2-3 proteinase responsible for processing at the NS2/3 junction [37-39]. Cleavage at this site most likely is an intramolecular and cotranslational reaction. Deletion studies show that most of the NS2 region and the amino terminal NS3 domain are required for efficient processing [37, 38, 67]. Within the NS2 sequence His-952 and Cys-993 are essential for enzymatic activity [37, 38]. This finding and the fact that enzymatic activity can be enhanced by zinc ions and reduced by chelating agents like EDTA led to the suggestion that the NS2-3 proteinase is a zinc-dependent metalloproteinase [38]. However, since zinc ions are also required for proper folding of the NS3 proteinase domain (see below), it is not known whether the activating effect is due to proper folding of the NS3 domain of the NS2-3 proteinase or whether zinc is also bound by NS2 and participates in the cleavage reaction.

The reason why the amino-terminal NS3 domain is required for cleavage at the NS2/3 junction is unknown as well. Either the enzymatic activity resides in the NS2 domain and the NS3 domain is required for proper folding of the proteinase or the NS2/3 cleavage site or both domains are required to constitute the enzymatically active proteinase. In the latter case, the NS2/3 site is present within the enzyme which would be inactivated after cleavage, reminiscent to the suicidal proteinase of the Sindbis virus capsid protein [for a review, see ref. 68].

Although the NS2-3 proteinase and the NS3 proteinase overlap, they carry independent activities because mutational inactivation of the NS2-3 enzyme only blocks cleavage at the NS2/3 site but does not affect processing at any of the NS3-dependent sites and inactivation of the NS3 proteinase has no effect on processing at the NS2/3 junction [28, 37, 38, 40]. However, at least for some HCV isolates, removal of the NS2 region from the NS3 domain appears to be required for efficient processing of the NS35B

region by the NS3 proteinase [69].

The NS3 Proteinase

The most intensively studied and therefore best understood target for antiviral therapy against HCV is the NS3 proteinase. Originally predicted on the basis of sequence comparisons between HCV and several other viral and cellular proteinases, the serine-type proteinase activity residing in the amino-terminal NS3 domain has been proven experimentally by several groups [28, 38, 40, 42]. It is required for cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NSSA/5B sites but dispensable for processing at all other known sites. The lack of detectable NS3/ 4A precursors, the insensitivity of cleavage kinetics at the NS3/4A site to dilution and the inability to cleave this site in trans strongly suggest that processing between NS3 and NS4A is an intramolecular and cotranslational reaction. In contrast, processing at the other NS3-dependent sites can be mediated in trans (table 1) [28, 40, 49, 51].

Several studies have demonstrated a preferential but not obligatory order of cleavages of the NS polyprotein precursor by the NS3 proteinase. The first, probably cotranslational, cleavage occurs between NS3 and NS4A and is followed by processing at the NS5A/5B junction. The resulting NS4A-5A intermediate is cleaved more slowly following different pathways [49-51, 70].

Deletion studies demonstrated that the amino-terminal 181 residues of NS3 are sufficient for full proteolytic activity [49, 51, 71, 72]. Further truncations at the carboxy terminus completely destroy the enzymatic activity, whereas removal of amino-terminal residues has a differential effect. While normal processing patterns are observed for NS3 proteinases lacking up to 14 amino-terminal residues, deletion of 22 amino acids severely reduces cleavage at the NS4A/4B site and completely blocks processing at the NS4Bl SA junction, whereas cleavage at the NS5A/5B site is only moderately affected [49, 71]. The reason for this differential effect will be given below.

First insights into the specificity of the enzyme were obtained from the determination of amino-terminal sequences of cleavage products. These studies allowed the precise localization of the processing sites [42, 73] and they led to the definition of the following consensus sequence: Asp/Glu-X-X-X-Cys/Thr (arrow down) Ser/Ala (where X is variable). An acidic residue is found at the P6 position, a P1-Cys residue at all trans-cleavage sites or a P1-Thr residue in case of the NS3/4A (cis) cleavage site and a residue with a small side chain is found at the P1' position. (According to Berger et al. [74], amino acids extending from the cleaved bond towards the amino terminus are denoted as P1, P2, P3, etc. and those extending towards the carboxy terminus are designated P1', P2', P3', etc.) Several studies have shown that the P1 residue is the primary determinant of substrate specificity whereas the P 1' and the P6 residues are highly tolerant towards substitutions [75-78]. Although these results imply that the acidic amino acid at the P6 position is less important for efficient substrate cleavage, one additional acidic residue is found on the P-side in close proximity to all NS3-dependent sites. As shown for the NS5A/5B site, substitution of both acidic residues blocks cleavage, suggesting that an overall negative charge in the region just upstream of the scissile bond is important for efficient processing [76].

For a given substitution, a site-dependent gradient in the inhibition was observed with the NS3/4A site showing the greatest tolerance and the NSSA/5B site being most sensitive [75-78]. This result probably reflects the different mechanisms operating at the NS3-dependent sites. Cleavage at the cis-site primarily seems to be governed by polyprotein folding bringing the proteinase in close contact to the substrate and compensating for a weak interaction with a less favourable P1 residue. In contrast, processing at the other trans-cleavage sites may be governed primarily by direct interaction between the proteinase and the polyprotein substrate requiring more stringent conditions.

The NS4A Proteinase Cofactor

Studies aimed at the identification of factors required in addition to NS3 for polyprotein cleavage demonstrated that the 54-residue NS4A is a proteinase cofactor [49-52], that is when NS3 is coexpressed with an NS4B-5B substrate in cell culture, only a low cleavage at the NS5A/5B site is found whereas processing at the NS4B/5A junction does not take place at all. However, when NS4A is coexpressed with the proteinase and the same substrate, efficient cleavage at both sites is restored. From this type of analysis it was shown that processing at the NS3/4A, NS4A/4B and NS4B/5A sites is absolutely dependent on NS4A whereas cleavage between NS5A and NS5B can be mediated by NS3 alone, although, at least for some HCV isolates, efficiency is significantly enhanced by the cofactor [50]. Proteinase activation can be achieved by NS4A expressed as part of the substrate or as part of the proteinase, but also when NS4A is expressed as a separate molecule in trans [49-51].

A first hint on how NS4A activates the NS3 proteinase came from immunoprecipitation studies showing that both molecules form a detergent-stable protein complex [26, 69, 79-81]. Disruption of the complex by amino acid substitutions within NS3 or NS4A also affects processing at the NS3-dependent sites, indicating that complex formation is an essential prerequisite for proteinase activation. This assumption also explains the differential effect observed for amino-terminal NS3 deletions described above. Removal of 14 residues does not affect processing at the NS3-dependent sites and has only a moderate effect on complex formation. In contrast, deletion of 22 amino acids severely affects cleavage at these sites and abolishes interaction with the cofactor. This result not only emphasizes the importance of complex formation for proteinase activation but also maps the NS4A binding domain within NS3 in the amino-terminal 22 residues [71, 79, 80, 82]. The only exception is cleavage at the NS3/4A site. In this case, a proteinase lacking 28 amino-terminal residues and unable to interact with the cofactor still can cleave at this site [71]. Possibly, processing at this cis-site is affected primarily by polyprotein folding, helping to place NS4A in the appropriate position for NS3-mediated cleavage. Therefore, an amino-terminally truncated NS3-4A precursor still may undergo self-cleavage because the cofactor is covalently linked to the proteinase, thereby compensating for a defective protein-protein surface.

Transient expression of constructs directing the expression of amino- and carboxy-terminally truncated NS4A proteins mapped a minimum proteinase activation region within the cofactor to the central domain from Gly21 to Ser-32 [52, 79, 80]. Since synthetic peptides carrying this region are sufficient for full proteinase activation, it constitutes an autonomous proteinase activation and binding domain [81, 83-86]. In summary, these results demonstrate that proteinase activation is mediated by complex formation for which amino-terminal NS3 sequences and the central region of NS4A are required.

However, certain mutations within these sequences which have no effect on proteinase activation reduce recovery of NS3/4A complexes, suggesting that a weak overall association between both proteins is sufficient for activation [71, 79, 80]. Perhaps the tight interaction observed between NS3 and NS4A serves another function, namely association of NS3 with intracellular membranes. NS4A is an amphipathic peptide tightly interacting with membranes via the hydrophobic amino terminus likely to form a transmembrane helix [26, 52]. Given that the carboxyterminal NS3 domain is an NTPase/helicase, membrane attachment of NS3 via NS4A might be required for the formation of a membrane-associated replicase complex.

Using several in vitro systems composed of NS4A-specific peptides, recombinant purified NS3 proteinase from different sources and peptide substrates or substrates generated by in vitro translation, the mechanism of NS4Amediated proteinase activation was analyzed. It was found that NS3 and the cofactor form a 1:1 complex suggesting that the enzymatically active proteinase is indeed a heterodimer [87, 88]. By interaction with NS4A, cleavage rates of the proteinase at the various sites are influenced to

very different extents. While cleavage efficiency (expressed as k^sub cat^/K^sub m^) is enhanced about 3-fold at the NS5A/5B site, an 11-fold increase is found at the NS4A/4B site and even a 100-fold increase at the NS4B/ 5A site [85, 88]. Thus, NS4A exerts its greatest effect at the site that is cleaved by the proteinase in the absence of the cofactor with the least efficiency. Interestingly, enhancement of cleavage at the NS5A/5B site and the NS4A/4B site is achieved essentially by an increase of k^sub cat^ (3-fold and 5-fold, respectively), whereas cleavage efficiency at the NS4B/5A site is enhanced by an increase of k^sub cat^ and K^sub m^ (16-fold and 6-fold, respectively) [88]. This increase of K^sub m for NS4B/5A substrates indicates a stabilizing effect of NS4A between the NS3 proteinase and the substrate and might explain the coprecipitation of NS4A with an uncleaved NS4B-5B substrate [79].

The way NS4A influences k^sub cat^ is not known. One explanation would be a conformational change in the NS3 proteinase after association with NS4A creating an optimal environment for catalysis. In agreement with this assumption Steinkuhler et al. [88] found that the physicochemical requirements (e.g. dependence on glycerol and detergents) necessary for optimal proteinase activity were different for an NS3 proteinase compared to an NS3/4A proteinase complex. Overall, these results suggest that NS4A exerts its activation function by (1) induction of conformational changes within NS3 and (2) stabilization of the proteinase/substrate interaction in case of the NS4B/5A site.

NS4A is not the only factor modulating NS3 proteinase activity. Using a purified full length NS3/4A expressed in mammalian cells, Morgenstern et al. [89] found that polynucleotides have a profound effect on enzymatic activity. When poly (U) was added to an in vitro transcleavage assay proteinase activity was enhanced about 5fold. Poly (A) and poly (C) had a lower activating effect (about twofold), whereas no significant enhancement was found for poly (G). Since no activation was found with a carboxy-terminally truncated NS3 proteinase lacking the NTPase/helicase domain, these results suggest that polynucleotides exert their activating effect on the proteinase indirectly via binding to the carboxy-terminal helicase domain. Although it is not clear whether poly (U) enhances catalytic activity or enzyme stability, in either case, the results imply an interdomain communication. This phenomenon also could explain why different enzymatic activities reside in one molecule. It is not simply for economical reasons but rather for mutual modulation or coupling of these activities.

Structure of the NS3/4A Proteinase Complex

Many of the biochemical studies described above were confirmed and extended by the recent resolution of the three-dimensional X-ray crystal structure of the NS3 proteinase domain [90] or the NS3 proteinase domain complexed with a synthetic NS4A peptide [91] (fig. 3). Both reports revealed a number of important and particular features of the HCV enzyme which will be listed below:

Fig. 3.

(1) In agreement with the experimentally determined inhibitor profile [92], the NS3 proteinase has a chymotrypsin-like fold with structural similarities to the proteinase residing in the Sindbis virus capsid protein.

(2) The NS3/4A proteinase is composed of two domains. The carboxy-terminal domain contains a sixstranded beta-barrel which is followed by a structurally conserved alpha-helix. The amino-terminal domain of NS3 contains 8 beta-strands with one contributed by NS4A (fig. 3). The active-site residues His-56 and Asp-79 are located in the amino-terminal domain, whereas the active-site residue Ser-139 resides in the carboxy-terminal domain and they project into the cleft which separates the 2 domains. The central NS4A proteinase activation sequence tightly intercalates into the amino-terminal domain essentially via hydrophobic interactions. Given this tight association, NS4A should be considered an integral component of the NS3/4A complex. In the absence of NS4A, the amino-terminal sequence of NS3 is loosely structured and extends away from the protein [90], suggesting that interaction with NS4A is required for correct folding of the sequence at the amino terminus of NS3. Such a reorganization of NS3 probably leads to a more stable structure explaining why NS4A binding to the proteinase inhibits NS3 degradation by cellular enzymes [52].

(3) A tetrahedrally coordinated zinc ion complexed via 3 Cys-residues and through a water molecule via one Hisresidue is present in the carboxy-terminal domain. Given the distance from the active site of the enzyme, zinc appears to have a structural and not a catalytic role. This finding explains the unusual sensitivity of the NS3 serinetype proteinase to divalent metal ions; e.g. it was found that Zn^sup 2+^ activates the enzyme whereas it is inhibited by Cu^sup 2+^ [93]. The assumption that Zn plays a structural role is further supported by two observations: (a) The amount of soluble NS3 proteinase expressed in Escherichia coli increases drastically when the cells are cultured in the presence of Zn-containing medium, whereas in the absence of this additive, the major part of NS3 aggregates [94]; (b) when the coordinating histidine residue is replaced by alanine and the protein is expressed in Escherichia coli, practically all protein accumulates in the insoluble fraction [95].

(4) Several loops involved in shaping the substrate binding pocket in case of structurally related chymotrypsin and elastase are missing, rendering the NS3 substrate binding pocket rather featureless and solvent exposed. The specificity pocket accommodating the P1 residue (designated the Sl pocket) is shallow, non-polar and formed primarily by the side chains of invariant residues Phe-154, Ala-157 and Leu-135. Substrate modelling studies [90] indicate that there are no distinct pockets for the P2-P5 side chains of the substrate and that this lack of interaction surface is compensated for by a continuous P2-P6 main-chain interaction. According to these predictions, the acidic P6 residue could interact with Arg-161 and Lys-165 of the NS3 proteinase domain.

Several studies have examined the substrate specificity of the NS3/4A proteinase in great detail. As described above, the Si pocket is formed primarily by 3 invariant residues. Of these, Phe-154 is of major importance. As indicated by homology modelling studies [73] and the resolution of the X-ray crystal structure [90, 91], Phe-154 is located at the bottom of the Si pocket, suggesting that this residue has a dual function: delimiting the length of the P1 residue that can fit into the pocket and establishing a favourable interaction between the correct P1 residue cysteine and the aromatic side chain of phenylalanine. This dual function might be the explanation that cysteine is the most favourable P 1 residue (and the only one found at the trans-cleavage sites). The importance of Phe-154 for substrate specificity was also demonstrated by mutation studies showing that alterations of this residue created enzymes with broader specificities [96, 97], e.g. substitution of Phe- 154 by threonine generated a proteinase accommodating a P1-Leu residue which was not accepted by the parental enzyme. For the other 2 amino acids forming the substrate binding pocket, a minor contribution of Ala-157 was found

whereas alterations of Leu-135 did not affect substrate specificity [97].

The way how substrate specificity is achieved was analyzed by Urbani and coworkers [98]. They found that synthetic peptide substrates which could not be cleaved or only very poorly still were bound efficiently by the proteinase. This result together with the modelling studies described above suggests that ground state binding of the substrate is mediated by multiple interactions involving in addition to the P1 amino acid also distal residues. The main function of the P 1 residue would be to determine the efficiency with which the bound substrate proceeds through the transition state. Both properties would by synergistic and generate the high specificity required to accomplish proper cleavage of the polyprotein substrate. Strategies for Inhibition of the NS3/4A Proteinase Identification of inhibitors targeted against the NS3/ 4A proteinase complex requires simple and reliable in vitro test systems which allow high volume 'random' screening of natural products, corporate collections of compounds or peptides and combinatorial chemical libraries. Several reports described the expression and, in some cases, purification of large amounts of enzymatically active NS3 or NS3/4A proteinase and they demonstrated in vitro cleavage of in vitro translated or recombinant HCV proteins or synthetic peptide substrates [8289, 92, 98-103]. These assays can now be used to pursue at least two different strategies of NS3-specific inhibition. First, inhibition of the catalytic mechanism of the NS3 proteinase and second, construction of dominant negative NS3 or NS4A peptides or compounds interfering with complex formation.

Concerning the first approach, rational drug design and high volume random screenings currently are pursued and first inhibitors have been identified [104]. However, one problem has to be considered which refers to the rather unstructured substrate binding pocket of NS3. As described above, in case of the HCV enzyme several loops shown for other proteinases to make essential contributions to substrate binding are missing. The S1 pocket is rather featureless and the P2-P5 residues appear to contact the enzyme primarily via main-chain interactions. This apparent lack of specificity of proteinase-substrate interaction may make the development of specific inhibitors rather difficult. On the other hand, the unusual substrate specificity which is quite distinct from cellular serine-type proteinases suggests that it should be possible to generate inhibitors with a high degree of selectivity. With the availability of the three-dimensional structure of the NS3 proteinase, rational drug design, which so far had to rely on structure modelling should be accelerated.

Concerning the interference of the NS3/4A interaction, several possibilities could be envisaged: (1) dominant negative NS4A peptides interacting with the substrate or blocking enzyme-substrate interaction; (2) NS3 peptides interacting with the cofactor in a way that it can no longer bind to the proteinase or (3) dominant negative NS4A peptides or synthetic compounds interacting with NS3 in a way that activation by the cofactor is no longer possible. Referring to this last approach, Shimizu et al. [85] have recently described an NS4A peptide in which Arg-28 was replaced by glutamine and this peptide inhibited proteinase activation in vitro. However, it should be kept in mind that in vivo, processing at the NS3/4A site most likely is an intramolecular and cotranslational event and is governed by a cotranslational interaction between the amino-terminal NS3 domain and the NS4A sequence. Given this rapid and efficient event, a posttranslational displacement of NS4A from the proteinase complex might be difficult to achieve. Obviously further studies are required to characterize proteinase-cofactor interaction and define of how proteinase activation is accomplished.

The Viral Replicase Complex: An Alternative Target for Drug Design

In the past few years, the mechanisms and enzymes involved in polyprotein processing of HCV have been a main focus of interest. Only recently have components of the viral replicase complex received more attention with the demonstration of enzymatic activity in the NS3 NTPase/helicase and the NS5B

RdRp. Both proteins are highly conserved among HCV strains and most likely essential for virus replication, making these enzymes further attractive targets for antiviral intervention.

The NS3 NTPase/Helicase

Helicases are enzymes capable of unwinding duplex DNA or RNA structures, i.e. generating single-stranded nucleic acids from a double-stranded substrate. This energy-dependent reaction is coupled to the hydrolysis of a nucleoside triphosphate (NTP) explaining why all helicases identified thus far also have an NTPase activity [for a recent review, see ref. 105]. Based on the existence of conserved amino acid sequence motifs, helicases have been divided into 3 superfamilies with superfamily 2 representing the NS3-like proteins of poty- and flaviviridae. The common denominator of these helicases is the NTP-binding motif composed of the A and the B site [106]. The A site is characterized by a stretch of hydrophobic amino acids followed by the conserved sequence GxxxxGKS/T (where x represents any residue). This site is directly involved in binding of the beta- and gamma-phosphates of the NTP. The B site also known as the 'DEAD' box (in case of HCV a 'DECH' box) serves to chelate the Mg^sup 2+^ of the Mg-NTP complex. Similar amino acid sequence motifs are found in the carboxy-terminal two thirds of the HCV NS3 and it is now well established that this protein domain has an NTPase/helicase activity [44-48, 107] catalyzing the hydrolysis of NTP and the unwinding of duplex RNA in the presence of divalent metal ions. Using purified recombinant protein and various in vitro assay systems the following properties of the enzyme have been observed:

(1) The minimal functional domain of the NTPase/ helicase is approximately 400 amino acids in length and maps between NS3 residues 1209 and 1608 [108].

(2) NS3 binds to homoribopolymers with the following order of specificity: poly(U) >> poly(A) > poly(C)/poly(G) [46]. Since the 3' NTR of the HCV genome contains a polyuridine stretch, NS3 might bind preferentially to the viral RNA [20-23, 109].

(3) Binding affinity also depends on the length of the nucleic acid which must have a minimum of 12-15 nucleotides [107]. This value is in a range comparable to the monomers of the E. coli Rep helicase and eukaryotic nuclear DNA helicase (16 nucleotides) [107 and references cited therein].

(4) The NTPase is relatively nonselective for the nucleoside and it can hydrolyze all ribonucleotides and deoxynucleotides [44, 107].

(5) NTPase activity is stimulated by polynucleotides up to 25-fold with polyuridine and polydeoxyuridine achieving maximal stimulation [44, 89, 107], whereas poly(A) and poly(C) are less effective. This stimulation is highly selective for the length of the nucleic acid and appears to induce a conformational change in the helicase [107].

(6) In contrast to the stimulation of NTPase, helicase activity is inhibited by polynucleotides with the following order of efficiency: poly(G) > poly(U) > poly(A) > poly(C) [48, 89, 107]. This inverse correlation between NTPase stimulation and inhibition of helicase activity suggests that polynucleotides compete with RNA substrates for the same or overlapping binding sites.

(7) The RNA unwinding activity has an absolute requirement for a divalent metal ion, either Mg2+ or Mn2+, and ATP suggesting a close coupling of this activity with ATP hydrolysis [107].

(8) The enzyme binds to substrates containing 3' or 5' single-stranded regions but not to blunt-ended RNAs. However, the RNA helicase unwinds RNA-RNA and DNA-DNA duplexes as well as

RNA-DNA heteroduplexes only in the 3' to 5' direction with respect to the template strand [46-48]. Given this directionality, the polarity of the RNA strand to which the helicase binds appears to be an important enzymatic determinant [46-48].

Very recently, the three-dimensional structure of the HCV NTPase/helicase domain was solved at 2.1 Angstrom resolution [110]. A 450-residue NS3 fragment was expressed as a histidine-tagged fusion protein in E. coli and after extensive purification and removal of the foreign sequence used for crystallization. It was found that the helicase has 3 nearly equal-sized domains resulting in a triangular molecule. The amino- and carboxy-terminal domains are closely packed and form one rigid unit. A deep groove separates this unit from the RNA-binding domain which contains several conserved arginine residues required for interaction with the nucleic acid. This RNA binding domain forms a rigid framework which is linked to the rest of the molecule rather flexibly. As deduced from comparisons of crystallographically independent molecules, the RNA-binding domain can rotate and this rotation is accomplished by concerted motions of an extended antiparallel beta-sheet and a highly conserved TATPP sequence also known as motif III in the helicase superfamily 2 [105]. Given the close proximity of the NTPase domain to the TATPP sequence, it is possible that structural rearrangements leading to rotation of the RNA binding domain are coupled to NTP hydrolysis.

In summary, these data show that NS3 of HCV is a polynucleotide-stimulated NTPase with biochemical characteristics shared by many other viral RNA helicases [105]. However, thus far, most studies were performed with truncated NS3 proteins comprising only the helicase domain. As shown by Morgenstern et al. [89], a full-length NS3 protein complexed with NS4A has some biochemical properties different from those described for truncated NS3. These differences include the pH value or the concentration of poly(U) required for optimal ATPase activity indicating that molecular interactions between the NS3/4A proteinase domain and the helicase domain probably affect ATPase activity of the latter. These results have to be kept in mind in that inhibitors designed against the helicase domain should also be evaluated in the context of an authentic NS3/4A protein complex.

The NS5B RdRp

The second obvious component of the viral replicase complex is the viral RdRp. Originally predicted on the basis of the 'GDD' motif to encode a polymerase, this enzymatic activity has now been proven [53-55]. Due to the difficulties in obtaining sufficient amounts of soluble enzymatically active NS5B in prokaryotic expression systems, most studies used recombinant baculoviruses. It was shown that NSSB is a phosphoprotein associated with intracellular membranes suggesting that HCV replication takes place in a membrane-bound replicase complex [111]. Two different enzymatic activities were described associated with purified NS5B: an RdRp and a terminal transferase (TNTase) adding one non-templated nucleotide to the 3' end of a substrate RNA [53]. As described for most viral RdRps, NS5B requires a primer to initiate RNA synthesis which at least under in vitro conditions is generated by intramolecularly base-paired, 3'-terminal sequences leading to the production of base-paired dimersized RNA molecules [53, 54]. This reaction is strongly preferred in that exogenous primers are only used when the 3'-OH group of the RNA template is blocked chemically [53, 54]. In case of homopolymeric templates which cannot form intramolecular base-pairings, RdRp activity strictly depends on the addition of exogenous primers which can be DNA or RNA [53, 54]. Whether initiation of RNA synthesis in the infected cell is also mediated by intramolecular priming is not known, but if it is, a specific ribonuclease would be required cleaving the RNA dimer at the initiation site.

Using an in vitro transcribed HCV full length RNA it was shown that the enzyme can copy a complete genome in the absence of additional cellular or viral proteins (fig. 4) [54]. Preliminary results from kinetic analyses indicate that the enzyme has an elongation rate of about 120 nucleotides per minute

which is slower compared to the values of 3D^sup Pol^ of poliovirus ranging between 300 and 1,250 nucleotides per minute [112, 113]. Whether this is an NS5B-inherent activity or due to the lack of an additional factor enhancing the catalytic activity remains to be determined.

The enzyme has no apparent template specificity and copies homologous and heterologous RNAs. This observation was substantiated with RNA binding studies and competition experiments [54]. Using an RNA corresponding to the last about 300 nucleotides of the HCV genome, it was found that binding of NS5B to this template could be competed equally well with homologous and heterologous RNAs. However, for homopolymeric templates a clear preference of binding was found: poly (U) > poly (G) > poly (A) > poly (C) [54]. Interestingly, an inverse order could be observed when these polymers were used as templates for the RdRp reaction. In this case maximal activity was found with poly (C)/oligo (G)^sub 12^, whereas poly (U)/oligo (A)^sub 12^ and poly (G)/oligo (C)^sub 12^ were inactive [54]. This result suggests that low binding of the polymerase to the primer and tight binding to the template correlates with low levels of RNA synthesis, whereas preferential binding to the primer and low binding of the template correlates with high levels of synthesis. Probably, tight binding of the polymerase to the template slows movement on it and inhibits RNA synthesis. A similar result has been described for 3D^sup POl^ of poliovirus [114].

Several studies have shown that nucleic acid polymerases show fundamental structural and mechanistic similarities which are reflected by distinct amino acid sequence motifs [for a review, see ref. 115]. These motifs, designated A-D according to the suggestions of Poch et al. [116], are also found in the central region of HCV NS5B (fig. 5A). Motif 'A' characterized by an invariant aspartic acid residue is most likely involved in nucleotide binding and catalysis. Motif 'B' carries an invariant glycine and probably plays a role in template and/or primer positioning. The 'GDD' motif, which is a hallmark of most polymerases, is part of motif 'C' involved in NTP binding and catalysis. Interestingly, within motif 'D' which also seems to be involved in NTP binding and catalysis, for all reverse transcriptases (RT) and nearly all viral RdRps, a lysine residue is found whereas an invariant arginine is present at this position in case of all HCV isolates (fig. 5A). The importance of these amino acids for enzymatic activity was recently shown by an intensive mutation analysis [54]. Numerous single substitutions were introduced into NS5B, expressed with recombinant baculoviruses in insect cells and purified to near homogeneity. None of the substitutions significantly affected RNA binding. However, with one exception, all amino acid exchanges severely reduced RdRp activity (fig. 5B). The exception was the mutation in which the arginine of motif 'D' was replaced by a lysine. This enzyme had an about 50% higher activity compared to the parental protein. As inferred from the three-dimensional structure of human immunodeficiency virus RT, this lysine together with the highly conserved carboxylates of motifs 'A' and 'C' is directly involved in catalysis [117, 118], suggesting that the arginine at this position directly reduces the enzymatic activity. Alternatively, the high activity of the altered enzyme could be explained by an indirect effect, e.g. by affecting the conformation increasing the number of enzymatically active molecules in the NS5B preparation.

In summary, the results confirm the importance of 4 amino acid motifs for RdRp activity. Since they are significant elements of the `polymerase module' constituting the framework of the enyzmatically active protein domain, it is likely that the central region of NSSB has structural similarities to those described for other polymerases and RTs. We can expect that activities or protein domains required for HCV-specific purposes reside in sequences amino- or carboxy-terminal of the central polymerase module.

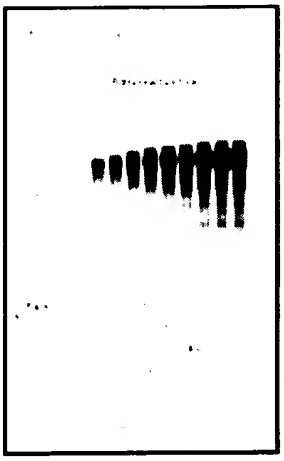

Fig. 4.

While it is obvious that the RdRp is essential for viral replication, the significance of the TNTase is less clear. As described above, an NS5B-associated activity adding a single ribonucleotide to the 3'-OH group of an RNA molecule was found with purified NS5B [53]. All 4 NTPs could be used as a substrate with a slight preference for UTP. The same enzymatic activity was described in a subsequent report, again associated with recombinant NS5B purified from insect cells [54]. However, in this case 2 observations suggested that the TNTase was due to a cellular protein copurifying in minute amounts with the HCV protein: (1) none of the 20 NS5B mutations reducing or blocking RdRp activity (fig. 5B) affected the TNTase activity and (2) it was also found with a purified NS4B protein and with lysates of cells infected with baculo wild type virus and purified in the same way. Finally, no TNTase was found with an NSSB expressed in E. coli (albeit within a rather low level of sensitivity [55]).

Although we are far away from a detailed understanding of the mechanisms underlying HCV replication, at least 2 viral proteins most likely important for this reaction could be identified: the NS3 NTPase/helicase and the NS5B RdRp. Both enzymes can be expressed in high quantities, they can be purified to homogeneity and appropriate in vitro assays are available. In case of the NS3 NTPase/helicase the three-dimensional structure was solved allowing a rational drug design. For the NS5B RdRp, the three-dimensional structure of the polymerase module e.g. from the HIV RT [117, 118] might be used as a guideline for homology modelling. These 'rational' approaches should facilitate and accelerate development of effective therapeutics targeted against components of the HCV replicase.

Conclusions and Future Directions

In the past few years our knowledge about the molecular biology of the HCV has increased rapidly and led to the identification of potential targets for antiviral intervention. As illustrated by the resolution of the X-ray crystal structure, the most advanced is the NS3/4A proteinase complex. However, despite this progress, several obstacles have to be overcome before effective therapeutics become available. The first refers to the high sequence variability of the HCV genome. This property has to be considered for at least two reasons. First, compounds are required which are effective against all different genotypes. Second, we can expect that therapy-resistant viruses will evolve. A combination therapy using drugs specific for different targets (e.g. NS3/4A proteinase and NS5B polymerase) might help to overcome this complication. Another issue to be considered concerns the lack of convenient animal models or appropriate cell culture systems to allow efficient virus propagation. This problem could be solved, at least to some extent, by using surrogate systems. For the NS3/4A proteinase several cell-based systems have been described allowing the evaluation of an intracellular inhibition of the enzyme [119, 120]. In addition, Hahm et al. [121] constructed a chimeric HCV/poliovirus in which the NS3 proteinase was

fused in frame to the amino terminus of the poliovirus polyprotein. Viability of this virus requires proteolytic removal of NS3 and, hence, inhibition of NS3 reduces virus formation. In a similar approach an HCV-Sindbis chimeric virus has been described whose replication and production of infectious progeny depends on the activity of the engineered NS3 proteinase [122]. Alternative to these systems, which depend on the replication of engineered viruses, the GBV-B virus [58] described above might be a useful model. As deduced from sequence comparisons and structure modelling, the GBV-B analogous NS3 proteinase is expected to have a substrate specificity similar to that of HCV. Since GBV-B efficiently replicates in tamarins (Sanguinus sp.), it might be used as a model to test for proteinase inhibitors in an animal. Another possible surrogate system would be transgenic animals expressing functional HCV proteins in a way that their inhibition can be easily monitored. However, it should be kept in mind that these systems do not allow firm conclusions on the inhibition of HCV replication in the infected cell. Irrespective of these limitations, the progress made during the last few years will keep us optimistic that effective antiviral drugs will become available in the not too far future.

Acknowledgments

Work in the author's laboratory was supported by grants from 'Bundesministerium fur Bildung, Wissenschaft, Forschung und Technologie' (01 KI 9653/9), `Deutsche Forschungsgemeinschaft' (Ba 1505/1-2) and by a grant from Roche Products Ltd., Welwyn Garden City, UK.

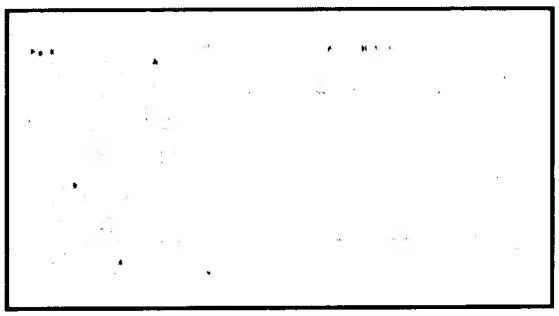

Fig. 5.

[Reference]

References

[Reference]

Houghton M: Hepatitis C viruses; in Fields BN, Knipe PM, Howley PM, et al (eds): Virology. Philadelphia, Lippincott-Raven Publishers, 1996, vol 2, pp 1035-1058. Cuthbert JA: Hepatitis C: Progress and problems. Clin Microbiol Rev 1994;7:505-532. Davis GL, Balart LA, Schiff ER, Lindsay K, Bodenheimer HC Jr, Perrillo RP, Carey W, Jacobson IM, Payne J, Dienstag JL, Van Thiel DH, Tamburro C, Lefkowitch J, Alberth J, Meschievitz C, Ortego TJ, Gibas A, and the Hepatitis Interventional Therapy Group: Treatment of chronic hepatitis C with recombinant interferon alfa: A multicenter randomized, controlled trial. N Engl J Med 1989;321: 1501-1506.

[Reference]

Marcellin P, Boyer N, Giostra E, Degott C, Courouce AM, Degos F, Cappere H, Cales P, Couzigou P, Benhamou JP: Recombinant human alpha-interferon in patients with chronic non-A, non-B hepatitis: A multicenter randomized controlled trial from France. Hepatology 199t;13:393-397.
Di Bisceglie AM, Shindo M, Fong TL, Fried MW, Swain MG, Gergasa NV, Axiotis CA, Waggoner JG, Park Y, Hoofnagle JH: A pilot study of ribavirin therapy for chronic hepatitis C. Hepatology 1992; 16:649-654. Kakumu S, Yoshioka K, Wakita T, Ishikawa T, Takayanagi M, Higashi Y: A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 1993; 105:507-512.
Reichard O, Yun ZB, Sonnerborg A, Weiland O: Hepatitis C viral RNA titres in serum prior to, during, and after oral treatment with ribavirin for chronic hepatitis C. J Med Virol 1993; 41:99-102.
Koskinas J, Tibbs C, Saleh MG, Pereira LM, McFarlane IG, Williams R: Effects of ribavirin on intrahepatic and extrahepatic expression of hepatitis C virus in interferon nonresponsive patients. J Med Virol 1995;45:29-34.

[Reference]

9 Lai M-Y, Kao J-H, Yang P-M, Wang J-T, Chen P-J, Chan K-W, Chu J-S, Chen D-S: Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. Gastroenterology 1996;111:1307-1312. 10 Choo Q-L, Weiner AJ, Overby LR, Kuo G, Houghton M, Bradley DW: Hepatitis C virus: The major causative agent of viral non-A, nonB hepatitis. Br

Med Bull 1990;46:423-441. 11 Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M: Isolation of a cDNA clone derived from a blood-borne nonA, non-B viral hepatitis genome. Science 1989; 244:359-362.
12 Inchauspe G, Yebedee S, Lee D-H, Sugitani M, Nasoff M, Prince A: Genomic structure of the human prototype strain H of hepatitis C virus: Comparison with American and Japanese isolates. Proc Natl Acad Sci USA 1991;88:1029210296.

[Reference]

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K: Molecular cloning of the human hepatitis C virus genome from Japanese patients with nonA, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524-9528.
Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J, Onishi E, Andoh T, Yoshida I, Okayama H: Structure and organization of the hepatitis C virus genome isolated from human carriers. J Virol 1991;65:11051113.

[Reference]

Okamoto H, Kojima M, Sakamoto M, Iizuka H, Hadiwandowo S, Suwignyo S, Miyakawa Y, Mayumi M: The entire nucleotide sequence and classification of a hepatitis C virus isolate of novel genotype from an Indonesian patient with chronic liver disease. J Gen Virol 1994;75: 629-635.
Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A: Internal ribosome entry site within hepatitis C virus RNA. J Virol 1992;66:14761483.

[Reference]

Wang C, Le S, Ali N, Siddiqui A: An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. RNA 1995;1:526-537.
Wang C, Sarnow P, Siddiqui A: Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J Virol 1993;67:3338-3344. Honda M, Ping L-H, Rijnbrand RC, Amphlett E, Clarke B, Rowlands D, Lemon SM: Structural requirements for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. Virology 1996;222:3142.

[Reference]

Tanaka T, Kato N, Cho M-J, Shimotohno K: A novel sequence found at the 3' terminus of hepatitis C virus genome. Biochem Biophys Res Commun 1995;215:744-749.
Kolykhalov AA, Feinstone SM, Rice CM: Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. J Virol 1996;70:3363-3371. Tanaka T, Kato N, Cho M-J, Sugiyama K, Shimotohno K: Structure of the 3' terminus of the hepatitis C virus genome. J Virol 1996;70: 3307-3312.
Yamada N, Tanihara NK, Takada A, Yorihuzi T, Tsutsumi M, Shimomura H, Tsuji T, Date T: Genetic organization and diversity of the 3' noncoding region of the hepatitis C virus. Virology 1996;223:255-261.
Hijikata M, Kato N, Ootsuyama Y, Nakagawa M: Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci USA 1991;88:5547-5551.
Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM: Expression and identification of hepatitis C virus polyprotein cleavage products. J Virol 1993;67:1385-1395.

[Reference]

26 Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, Akagi T, Kato N, Kimura K, Shimotohno K: Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc Natl Acad Sci USA 1993;90:10733-10777. 27 Selby MJ, Choo Q-L, Berger K, Kuo G, Glazer E, Eckart M, Lee C, Chien D, Kuo C, Houghton M: Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. J Gen Virol 1993;74: 103-1113.

[Reference]

28 Tomei L, Failla C, Santolini E, De Francesco R, La Monica N: S3 is a serine protease required for processing of hepatitis C virus polyprotein. J Virol 1993;67:4017-4026. 29 Lin C, Lindenbach BD, Pragai BM, McCourt DW, Rice CM: Processing in the hepatitis C virus E2-NS2 region: Identification of p7 and two distinct El-specific products with different C termini. J Virol 1994;68:5063-5073. 30 Mizushima H, Hijikata M, Asabe S-I, Hirota M, Kimura K, Shimotohno K: Two hepatitis C virus glycoprotein E2 products with different C termini. J Virol 1994;68:6215-6222. 31 Santolini E, Migliaccio G, La Monica N: Biosynthesis and biochemical properties of the hepatitis C virus core protein. J Virol 1994;68: 3661-3641.
32 Matsumoto M, Hwang SB, Jeng K-S, Zhu N, Lai MM: Homotypic interaction and multimerization of hepatitis C virus core protein. Virology 1996;218:43-51.
33 Ralston R, Thudium K, Berger K, Kuo C, Gervase B, Hall J, Selby M, Kuo G, Houghton M, Choo Q-L: Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J Virol 1993; 67:6753-6761.
34 Spaete RR, Alexander D, Rugroden M, Choo Q-L, Berger K, Crawford K, Kuo C, Leng S, Lee C, Ralston R, Thudium K, Tung JW, Kuo G, Houghton M: Characterization of the hepatitis C virus E2/NS I gene product expressed in mammalian cells. Virology 1992;188:819830.

[Reference]

35 Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, Rice CM: Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and sindbis viruses. J Virol 1994;68:6147-6160.
36 Selby MJ, Glazer E, Masiarz F, Houghton M: Complex processing and protein:protein interactions in the E2:NS2 region of HCV. Virology 1994;204:114-122.
37 Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM: A second hepatitis C virus-encoded proteinase. Proc Natl Acad Sci USA 1993;90:10583-10587.
38 Hijikata M, Mizushima H, Akagi T, Mori S, Kakiuchi N, Kato N, Tanaka T, Kimura K, Shimotohno K: Two distinct proteinase

activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J Virol 1993;67:4665-4675.

[Reference]

39 Hirowatari Y, Hijikata M, Tanji Y, Nyunoya H, Mizushima H, Kimura K, Tanaka T, Kato N, Shimotohno K: Two proteinase activities in HCV polypeptide expressed in insect cells using baculovirus vector. Arch Virol 1993;133: 349-356.

[Reference]

Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H: Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J Virol 1993;67:3835-3844. Eckart MR, Selby M, Masiarz F, Lee C, Berger K, Crawford C, Kuo C, Kuo G, Houghton M, Choo Q-L: The hepatitis C virus encodes a serine proteinase involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. Biochem Biophys Res Commun 1993;192:399-406.
Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM: Characterization of the hepatitis C virus-encoded serine proteinase: Determination of proteinase-dependent polyprotein cleavage sites. J Virol 1993;67:28322843.

[Reference]

Manabe S, Fuke I, Tanishita O, Kaji C, Gomi Y, Yoshida S, Mori C, Takamizawa A, Yosida I, Okayama H: Production of nonstructural proteins of hepatitis C virus requires a putative viral protease encoded by NS3. Virology 1994; 198:636-644.
Suzich JA, Tamura JK, Palmer-Hill F, Warrener P, Grakoui A, Rice CM, Feinstone SM, Collett MS: Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with related pestivirus and flavivirus enzymes. J Virol 1993;67:61526158.

[Reference]

Kim DW, Gwack Y, Han JH, Choe J: C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase. Biochem Biophys Res Commun 1995;215:160-166. Gwack Y, Kim DW, Han JH, Choe J: Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. Biochem Biophys Res Commun 1996; 225:654-659.
Hong Z, Ferrari E, Wright-Minogue J, Chase R, Risano C, Seelig G, Lee C-G, Kwong AD: Enzymatic characterization of hepatitis C virus NS3/4A complexes expressed in mammalian cells by using the herpes simplex virus amplicon system. J Virol 1996;70:4261-4268. Tai C-L, Chi W-K, Chen D-S, Hwang LH: The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). J Virol 1996; 70:8477-8484.
Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H: Kinetic and structural analyses of hepatitis C virus polyprotein processing. J Virol 1994;68:5045-5055.
Failla C, Tomei L, De Francesco R: Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J Virol 1994;68:3753-3760.
Lin C, Pragai BM, Grakoui A, Xu J, Rice CM: Hepatitis C virus NS3 serine proteinase: Transcleavage requirements and processing kinetics. J Virol 1994;68:8147-8157.

[Reference]

Tanji Y, Hijikata M, Satoh S, Kaneko T, Shimotohno K: Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J Virol 1995;69: 1575-1581.
Behrens S-E, Tomei L, De Francesco R: Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J 1996;15:12-22.
Lohmann V, Korner F, Herian U, Bartenschlager R: Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol 1997;71:8416-8428.
Yuan ZH, Kumar U, Thomas HC, Wen YM, Monjardino J: Expression, purification, and partial characterization of HCV RNA polymerase. Biochem Biophys Res Commun 1997; 232:231-235.
Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH: Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. Science 1985; 229:726-733.
Meyers G, Thiel H-J: Molecular characterization of pestiviruses. Adv Virus Res 1996;47: 53-118.

[Reference]

58 Muerhoff AS, Leary TP, Simons JN, PilotMatias TJ, Dawson GJ, Erker JC, Chalmers ML, Schlauder GG, Desai SM, Mushawar IK: Genomic organization of GB viruses A and B: Two new members of the flaviviridae associated with GB agent hepatitis. J Virol 1995;69: 5621-5630.
59 Miller RH, Purcell RH: Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus subgroups. Proc Natl Acad Sci USA 1990;87:2057-2061.
60 Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (eds): Classification and nomenclature of viruses. Sixth report of the international committee on taxonomy of viruses. Wien, Springer, 1995, pp 424-426. 61 Zignego AL, Macchia D, Monti M, Thiers V, Mazzetti M, Foschi M, Maggi E, Romagnani S, Gentilini P, Brechot C: Infection of peripheral mononuclear blood cells by hepatitis C virus. J Hepatol 1992;15:382-386.
62 Lumbreras C, Delgado R, Fuertes A, Loinaz C, Iglesias J, Colina F, Aguado JM, Gimeno C, Garcia I, Lizasoain M, Moreno E, Noriega AR: Clinical significance of hepatitis C virus (HCV) infection in liver transplant recipients - role of serology and HCV RNA detection. Dig Dis Sci 1994;39:965-969.
63 Martell M, Esteban JI, Quer J, Vargas V, Esteban R, Guardia J, Gomez J: Dynamic behaviour of hepatitis C virus quasispecies in patients undergoing orthotopic liver transplantation. J Virol 1994;68:3425-3436. 64 Mizushima H, Hijikata M, Tanji Y, Kimura K, Shimotohno K: Analysis of N-terminal processing of hepatitis C virus nonstructural protein 2. J Virol 1994;68:2731-2734.

[Reference]

65 Lohmann V, Koch JO, Bartenschlager R: Processing pathways of the hepatitis C virus proteins. J Hepatol 1996;24(suppl 2):11-19. 66 Hssy P, Langen H, Mous J, Jacobsen H: Hepatitis C virus core protein: Carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 1996;224:93-104.
67 Santolini E, Pacini L, Fipaldini C, Migliaccio G, La Monica N: The NS2 protein of hepatitis C virus is a transmembrane polypeptide. J Virol 1995;69:7461-7471.
68 Strauss JH, Strauss EG: The alphaviruses: Gene expression, replication, and evolution. Microbiol Rev 1994;58:491-562.
69 D'Souza ED, O'Sullivan E, Amphlett EM, Rowlands DJ, Sangar DV, Clarke BE: Analysis of NS3-mediated processing of the hepatitis C virus non-structural region in vitro. J Gen Virol 1994;75:3469-3476.
70 Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K: Hepatitis C virus polyprotein processing: Kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J Virol 1994;68: 8418-8422.

[Reference]

71 Failla C, Tomei L, De Francesco R: An aminoterminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. J Virol 1995:69:1769-1777.
72 Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K: Identification of the domain required from trans-cleavage activity of hepatitis C viral serine proteinase. Gene 1994;145:215-219. 73 Pizzi E, Tramontano A, Tomei L, La Monica N, Failla C, Sardana M, Wood T, De Francesco R: Molecular model of the specificity pocket of the hepatitis C virus protease: Implications for substrate recognition. Proc Natl Acad Sci USA 1994;91:888-892.
74 Berger A, Schechter I: Mapping the active site of papain with the aid of peptide substrates and inhibitors. Phil Proc R Soc London B 1970; 257:249-264.
75 Kolykhalov AA, Agapov EV, Rice CM: Specificity of the hepatitis C virus NS3 protease: Effects of substitutions at the 3/4A, 4A/4B, 4B/ 5A, and 5A/5B cleavage sites on polyprotein processing. J Virol 1994;68:7525-7533. 76 Komoda Y, Hijikata M, Sato S, Asabe S-I, Kimura K, Shimotohno K: Substrate requirements of hepatitis C virus serine proteinase for intermolecular polypeptide cleavage in Echerichia coli J Virol 1994;68:7351-7357. 77 Leinbach SS, Bhat RA, Xia S-M, Hum W-T, Stauffer B, Davis AR, Hung PP, Mizutani S: Substrate specificity of the NS3 serine proteinase of hepatitis C virus as determined by mutagenesis at the NS3/NS4A junction. Virology 1994;204:163-169.
78 Bartenschlager R, Ahlborn-Laake L, Yasargil K, Mous J, Jacobsen H: Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. J Virol 1995;69: 198-205.

[Reference]

79 Bartenschlager R, Lohmann V, Wilkinson T, Koch JO: Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. J Virol 1995;69:7519-7528.

[Reference]

80 Lin C, Thomson JA, Rice CM: A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J Virol 1995;69:4273-4380.
81 Satoh S, Tanji Y, Hijikata M, Kimura K, Shimotohno K: The N-terminal region of hepatitis C virus nonstructural protein 3 (NS3) is essential for stable complex formation with NS4A. J Virol 1995;69:4255-4260. 82 Koch JO, Lohmann V, Henan U, Bartenschlager R: In vitro studies on the activation of the hepatitis C virus NS3 proteinase by the NS4A cofactor. Virology 1996;221:54-66. 83 Lin C, Rice CM: The hepatitis C virus NS3 serine proteinase and NS4A cofactor: Establishment of a cell-free trans-processing assay. Proc Natl Acad Sci USA 1995;92:7622-7626. 84 Butkiewicz NJ, Wendel M, Zhang R, Jubin R, Pichardo J, Smith EB, Hart AM, Ingram R, Durkin J, Mui PW, Murray MG, Ramanathan L, Dasmahapatra B: Enhancement of hepatitis C virus NS3 proteinase activity by association with NS4A-specific synthetic peptides: Identification of sequence and critical residues of NS4A cofactor activity. Virology 1996;225: 328-338.

[Reference]

85 Shimizu Y, Yamaji K, Masuho Y, Yokota T, Inoue H, Sudo K, Satoh S, Shimotohno K: Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. J Virol 1996;70:127-132.
85 Tomei L, Failla C, Vitale RL, Bianchi E, De Francesco R: A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. J Gen Virol 1996;77:1065-1070. 87 Steinkuhler C, Tomei L, De Francesco R: In vitro activity of hepatitis C virus protease NS3 purified from recombinant baculovirus-infected Sf9 cells. J Biol Chem 1996;271:63676373.

[Reference]

88 Steinkuhler C, Urbani A, Tomei L, Biasiol G, Sardana M, Bianchi E, Pessi A, De Francesco R: Activity of purified hepatitis C virus protease NS3 on peptide substrates. J Virol 1996; 70:6694-6700.
89 Morgenstern KA, Landro JA, Hsiao K, Lin C, Gu Y, Su MS-S, Thomson JA: Polynucleotide modulation of the protease, nucleoside triphosphatase and helicase activities of a hepatitis C virus NS3-4A complex isolated from transfected COS cells. J Virol 1997;71:3767-3775. 90 Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW, Adachi T, Hostomska Z: The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell 1996;87:331-342.
91 Kim JL, Morgenstern KA, Lin C, Fox T, Dwyer MD, Landro JA, Chambers SP, Markland W, Lepre CA, O'Malley ET, Harbeson SL, Rice CM, Murcko MA, Caron PR, Thomson JA: Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell 1996;87: 343-355.

[Reference]

92 Hahm B, Han DS, Back SH, Song O-K, Cho M-J, Kim C-J, Shimotohno K, Jang SK: NS34A of hepatitis C virus is a chymotrypsin-like protease. J Virol 1995;69:2534-2539. 93 Han DS, Hahm B, Tho H-M, Jang SL: Identification of the

protease domain in NS3 of hepatitis C virus. J Gen Virol 1995;76:985993.

[Reference]

94 De Francesco R, Urbani A, Nardi MC, Tomei L, Steinkuhler C, Tramontano A: A zinc binding site in viral serine proteinases. Biochemistry 1996;35:13282-133287.
95 Stempniack M, Hostomska Z, Nodes B, Hostomsky Z: The NS3 proteinase domain of hepatitis C virus is a zinc-containing enzyme. J Virol 1997;71:2881-2886.
96 Failla CM, Pizzi E, De Francesco R, Tramontano A: Redesigning the substrate specificity of the hepatitis C virus NS3 protease. Folding Design 1996;1:35-42.
97 Koch JO, Bartenschlager R: Determinants of substrate specificity in the NS3 serine-proteinase of the hepatitis C virus. Virology 1997;237:78-88.
98 Urbani A, Bianchi E, Narjes F, Tramontano A, De Francesco R, Steinkhler C, Pessi A: Substrate specificity of the hepatitis C virus serine protease NS3. J Biol Chem 1997;272: 9204-9209.

[Reference]

99 Bouffard P, Bartenschlager R, Ahlborn-Laake L, Mous J, Roberts N, Jacobsen H: An in vitro assay for hepatitis C virus NS3 serine proteinase. Virology 1995;209:52-59. 100 Kakiuchi N, Hijikata M, Komoda Y, Tanji Y, Hirowatari Y, Shimotohno K: Bacterial expression and analysis of cleavage activity of HCV serine proteinase using recombinant and synthetic substrate. Biochem Biophys Res Commun 1995;210:1059-1065. 101 Overton, McMillan D, Gillespie F, Mills J: Recombinant baculovirus-expressed NS3 proteinase of hepatitis C virus shows activity in cell-based and in vitro assays. J Gen Virol 1995;76:3009-3019.
102 Shoji I, Suzuki T, Chieda S, Sato M, Harada T, Chiba T, Matsuura Y, Miyamura T: Proteolytic activity of NS3 serine proteinase of hepatitis C virus efficiently expressed in Escherichia coli. Hepatology 1995;22:16481654.

[Reference]

103 Suzuki T, Sato M, Chieda S, Shoji I, Harada T, Yamakawa Y, Watabe S, Matsuura Y, Miyamura T: In vivo and in vitro trans-cleavage activity of hepatitis C virus serine proteinase expressed by recombinant baculoviruses. J Gen Virol 1995;76:3021-3029. 104 Chu M, Mierzwa R, Truumees I, King A, Patel M, Berrie R, Hart A, Butkiewicz N, Dasmahapatra B, Chan TM, Puar MS: Structure of SCH 68631: A new hepatitis C virus proteinase inhibitor from Streptomyces sp. Tetrahedron Lett 1996;40:7229-7232. 105 Kadar* G, Haenni A-L: Virus-encoded RNA helicases. J Virol 1997;71:2583-2590.

[Reference]

106 Walker JE, Saraste M, Runswick MJ, Gay NJ: Distantly related sequences in the a- and bsubunits of ATP synthetase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. Embo J 1982;1: 945-951.
107 Preugschat F, Averett DR, Clarke BE, Porter DJT: A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 hellicase domain. J Biol Chem 1996;271:2444924457.

[Reference]

108 Kim DW, Gwack Y, Han JH, Choe J: Towards defining the minimal functional domain for NTPase and RNA helicase activities of the hepatitis C virus NS3 protein. Virus Res 1997;49:17-25.
109 Kanai A, Tanabe K, Kohara: Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. FEBS Lett 1995;376: 221-224.
110 Yao NH, Hesson T, Cable M, Hong Z, Kwong AD, Le HV, Weber PC: Structure of the hepatitis C virus RNA helicase domain. Nat Struct Biol 1997;4:463-467.

[Reference]

Hwang SB, Park K-J, Kim Y-S, Sung YC, Lai MMC: Hepatitis C virus NSSB protein is a membrane-associated phosphoprotein with a predominantly perinuclear localization. Virology 1997;227:439-446.
Neufeld KL, Richards OC, Ehrenfeld E: Purification, characterization, and comparison of poliovirus RNA polymerase from native and recombinant sources. J Biol Chem 1991;266: 24212-24219.
Van Dyke TA, Rickles RJ, Flanegan JB: Genome-length copies of poliovirion RNA are synthesized in vitro by the poliovirus RNAdependent RNA polymerase. J Biol Chem 1982;257:4610-4617.
Oberste MS, Flanegan JB: Measurement of poliovirus RNA polymerase binding in poliovirion and nonviral RNA using a filter-binding assay. Nucl Acids Res 1988;16:1033910341.

[Reference]

115 Joyce CM, Steitz TA: Polymerase structures and function: Variations on a theme? J Bacteriology 1995;177:6321-6329.
116 Poch O, Sauvaget I, Delarue M, Tordo N: Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. Embo J 1989;8:3867-3874. 117 Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA: Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science 1992;256: 1783-1790.

[Reference]

Jacobo-Molino A, Ding J, Nanni RG, Clark AD Jr, Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P, Hizi A, Hughes SH, Arnold E: Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. Proc Natl Acad Sci USA 1993;90:6320-6324. Hirowatari Y, Hijikata M, Shimotohno K: A novel method for the analysis of viral proteinase activity encoded by hepatitis C virus in cultured cells. Anal Biochem 1995;225:113120.

[Reference]
120 Song O, Cho OH, Hahm B, Jang SK: Development of an in vivo assay system suitable for screening inhibitors of hepatitis C viral protease. Mol Cells 1996;6:183-189. 121 Hahm B, Back SH, Lee TG, Wimmer E, Jang SK: Generation of a novel poliovirus with requirement of hepatitis C virus protease NS3 activity. Virology 1996;226:318-326. 122 Filocamo G, Pacini L, Migliaccio G: Chimeric Sindbis viruses dependent on the NS3 protease of hepatitis C virus. J Virol 1997;71: 1417-1427.

[Author note]
Ralf Bartenschlager
Institute for Virology, University of Mainz
Ober Zahlbacher Strasse 67
D-55131 Mainz (Germany)
Tel. +49 6131 174451, Fax +49 6131 395604, E-Mail bartnsch@mzdmza.zdv.uni-mainz.de

---